

Elicitor-Stimulated Furanocoumarin Biosynthesis in Cultured Parsley Cells: S-Adenosyl-L-Methionine: Bergaptol and S-Adenosyl-L-Methionine: Xanthotoxol O-Methyltransferases

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Dedicated to Professor Hans Grisebach on the occasion of his 60th birthday

Furanocoumarins, Phytoalexins, O-Methyltransferases, Fungal Elicitor, Cell Suspension Cultures

Cultured parsley cells (*Petroselinum crispum*) treated with an elicitor from the soybean pathogen, *Phytophthora megasperma* f. sp. *glycinea*, were used as source for the separation, extensive purification and characterization of S-adenosyl-L-methionine:xanthotoxol and S-adenosyl-L-methionine:bergaptol O-methyltransferases. The products, xanthotoxin and bergapten, were among the most abundant coumarin derivatives accumulated in the culture fluid of elicitor-treated parsley cells. The latter enzyme also catalyzed the methylation of 5-hydroxyxanthotoxin to isopimpinellin, another major coumarin derivative in this system. The activities of both enzymes showed transient increases upon elicitor application prior to the accumulation of the products. No activity was detectable in untreated cells. Activity increases of the O-methyltransferases occurred a few hours later than those of phenylalanine ammonia-lyase and 4-coumarate:CoA ligase.

Introduction

Coumarins are common constituents of many species of the Apiaceae. Pathogen-infected plants of a number of species contain increased levels of coumarin derivatives [1]. Among the Apiaceae, parsnip and celery have been shown to accumulate additional amounts of furanocoumarins in response to pathogen attack [2–4]. Handling of celery infected with *Sclerotinia sclerotiorum* has been reported to cause skin disorders through the action of linear furanocoumarins accumulating in the tissue of diseased plants [5]. Cultured parsley cells, which do not normally produce detectable amounts of coumarins, respond to treatment with fungal elicitors with the production of furanocoumarins and a dihydropyranocoumarin, the putative phytoalexins of this species [6].

Abbreviations: BMT: S-Adenosyl-L-methionine:bergaptol O-methyltransferase; C4H: Cinnamate 4-hydroxylase (EC 1.14.13.11); 4CL: 4-Coumarate:CoA ligase (EC 6.2.1.12); CMT: S-Adenosyl-L-methionine:caffate O-methyltransferase; DUD: Dimethylallyl diphosphate:umbelliferone dimethylallyltransferase; FPLC: Fast protein liquid chromatography; HPLC: High performance liquid chromatography; PAL: Phenylalanine ammonia-lyase (EC 4.3.1.5); SAH: S-Adenosyl-L-homocysteine; SAM: S-Adenosyl-L-methionine; TLC: Thin-layer chromatography; XMT: S-Adenosyl-L-methionine:xanthotoxol O-methyltransferase.

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Furanocoumarins are synthesized from phenylalanine *via* cinnamate, 4-coumarate, umbelliferone, and psoralen [1]. The activities of the enzymes of general phenylpropanoid metabolism, phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate:CoA ligase (4CL), and of one enzyme of the furanocoumarin pathway, dimethylallyl diphosphate:umbelliferone dimethylallyltransferase (DUD), have been shown to be stimulated by elicitor application to parsley cells [7–9]. The activity increases of PAL and 4CL were found to be due to transient activation of the respective genes [10, 11].

In connection with our studies of the mechanism of defense-related gene activation in plants, we report here the purification and characterization of two O-methyltransferases from elicitor-treated parsley cells, which specifically catalyze final methylation steps in the biosynthesis of three furanocoumarins, xanthotoxin, bergapten, and isopimpinellin.

Materials and Methods

Chemicals

Umbelliferone, esculetin, scopoletin, herniarin, psoralen, bergapten, xanthotoxol, xanthotoxin, and isopimpinellin were purchased from Roth (Karlsruhe, FRG). Isoimperatorin, daphnetin, hydrangeatin, 8-hydroxybergapten, 5-hydroxyxanthotoxin, and 5,8-dihydroxy-psoralen were provided by S. A.



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Brown (Peterborough, Canada), and 4-hydroxycoumarin, 2,4,6-trihydroxybenzoate, quercetin, and kaempferol by W. Barz (Münster, FRG). Molecular weight standards for gel filtration were from Boehringer (Mannheim, FRG) and for SDS gel electrophoresis from BRL (Neu Isenburg, FRG). Sephadex G-100 and a Mono Q HR 5/5 column were purchased from Pharmacia (Freiburg, FRG), ω -AH-Sepharose 4B from Sigma (München, FRG), DEAE-Cellulose DE 52 from Whatman (Watford, England), Dowex 1X2, 200–400 mesh, from Serva (Heidelberg, FRG), and Ultrogel AcA 44 from LKB (Gräfelfing, FRG). S-Adenosyl-L-methionine (SAM) and S-adenosyl-L-homocysteine (SAH) were from Sigma (München, FRG) and S-adenosyl-L-[methyl- 14 C]methionine (51–60 mCi/mmol) from Amersham-Buchler (Braunschweig, FRG). All other chemicals were of analytical grade and were purchased from Sigma (München, FRG) or Merck (Darmstadt, FRG).

Bergaptol was synthesized from isoimperatorin [12] and identified by mass spectrometry and after enzymatic methylation by co-chromatography with authentic bergapten on thin-layer chromatograms developed in solvent systems 1, 2, and 3.

Thin-layer chromatography

The following solvent systems were used for thin-layer chromatography (TLC) on silica gel 60 and silica gel 60F₂₅₄ (Merck, Darmstadt, FRG): 1. toluene/ethylformiate/formiate, 5:4:1 (v/v/v), 2. chloroform, 3. cyclohexane/chloroform, 1:2 (v/v), and 4. benzene/acetate, 4:1 (v/v). Solvent systems 1, 2, and 3 were used for analyses of coumarin derivatives, solvent system 4 for separation of caffeate and ferulate. Furanocoumarins were detected by their fluorescence under UV light at 366 nm, all other compounds under UV light at 254 nm by fluorescence quenching on TLC plates containing fluorescence indicator.

High performance liquid chromatography

Reversed-phase high performance liquid chromatography (HPLC) was carried out on a Zorbax ODS column (Du Pont, Bad Nauheim, FRG). Water containing 2% acetic acid/tetrahydrofuran, 83:17 (v/v) was used as solvent for isocratic chromatography. Similar separation of coumarin derivatives was obtained with a linear gradient from

2% aqueous acetic acid/tetrahydrofuran, 93:7 (v/v) to 2% aqueous acetic acid/methanol/tetrahydrofuran, 1:16:3 (v/v/v) within 20 min. In both cases the flow rate was 1.5 ml/min and the column temperature 40 °C. Coumarin derivatives were detected by their absorbance at 300 nm and by their fluorescence (excitation 330 nm, emission 480 nm). A Du Pont HPLC system, series 8800 (Du Pont, Bad Nauheim, FRG), was used with an automatic sample injection system, WISP 710 (Waters, Königstein, FRG), and with a fluorescence spectromonitor RF-530 (Shimadzu, Düsseldorf, FRG). Coumarin derivatives were identified by co-chromatography with authentic compounds and by their characteristic absorbance/fluorescence ratios. Psoralen, xanthotoxin, bergapten, and isopimpinellin were quantified by comparison with known amounts of authentic compounds using the isocratic system.

Fungal cultures and elicitor preparation

Phytophthora megasperma f. sp. *glycinea*, race 1, was grown as described by Kombrink and Hahlbrock [13]. Elicitor was prepared after Ayers *et al.* [14].

Plant cell cultures and elicitor treatment

Cell suspension cultures of parsley (*Petroselinum crispum*) recently derived from leaf petioles [11] were grown in the dark on modified B5 medium [15] as described elsewhere [13]. After six days of growth the cells were treated with elicitor (50 μ g/ml) under sterile conditions [13]. In large-scale experiments for enzyme purification, the elicitor was replaced by 0.1 ml fungal culture filtrate per ml cell culture. The cells were harvested by filtration under reduced pressure, frozen in liquid nitrogen, and stored at –80 °C. Cells used for purification of XMT and BMT were harvested 50 h after treatment.

Extraction of coumarins

Coumarin derivatives were extracted from culture fluid or cells [13], and the total amounts were monitored by UV absorption at 320 nm [13] and calculated using an average molar extinction coefficient of 12,000 l/mol.cm. The same extracts were used for HPLC and TLC analyses.

Enzyme extraction

PAL and 4CL were extracted as described [13]. For XMT and BMT extraction, frozen cells were

ground to powder in a mortar with liquid nitrogen and quartz (50% of cell fresh weight [w/w]). Per g cell fresh weight, 2 ml of 100 mmol/l potassium phosphate buffer, pH 7.0, containing 5 mmol/l ethylenediamine tetraacetate (EDTA), 10 mmol/l 2-mercaptoethanol, and 1 g Dowex 1 X2 (equilibrated with the same buffer), were added and the solution was stirred for 20 min at 4 °C. After filtration through miracloth, the extract was centrifuged for 30 min at $48,000 \times g$. All treatments in buffer were carried out at 4 °C.

Enzyme assays

PAL and 4CL were assayed as described elsewhere [13]. The standard assay for SAM:xanthotoxol O-methyltransferase (XMT) activity contained 100 mmol/l potassium phosphate, pH 7.5, 20 mmol/l ascorbate, 1.5 mmol/l MgCl_2 , 0.2 mmol/l xanthotoxol in 5 μl ethyleneglycol monomethylether, 4.2–4.9 $\mu\text{mol/l}$ [$^{14}\text{CH}_3$]SAM (0.05 μCi), 5 $\mu\text{mol/l}$ SAM, and 50 μl enzyme solution in a total volume of 200 μl . SAM:Bergaptol O-methyltransferase (BMT) activity was determined by the same method, except that xanthotoxol was replaced by 0.25 mmol/l bergaptol and the pH was adjusted to 8.0. In assays for SAM:caffeate O-methyltransferase (CMT) activity the pH was 7.5 and 1 mmol/l caffeate was used as substrate.

The enzyme reactions were terminated by addition of 30 μl 4 N HCl. Products were extracted with 200 μl ethylacetate containing 0.1 mg/ml xanthotoxin or bergapten or 0.2 mg/ml ferulate as carrier substances. Portions of the extracts were used for product identification on TLC and for the determination of radioactivity by liquid scintillation spectrometry.

Enzyme purification

All purification steps except fast protein liquid chromatography (FPLC) were performed at 4 °C. XMT and BMT were precipitated from the crude extract with 40–70% ammonium sulfate. Precipitated protein was dissolved in 20 mmol/l potassium phosphate, pH 7.0, containing 10 mmol/l 2-mercaptoethanol. The solution was desalted by dialysis against the same buffer and clarified by centrifugation at $25,000 \times g$ for 10 min.

DEAE-Cellulose. The resulting protein solution was chromatographed on DEAE-Cellulose (30×2.6 cm) which had been equilibrated with

20 mmol/l potassium phosphate, pH 7.0, and 10 mmol/l 2-mercaptoethanol. After elution of unbound protein with 150 ml of the same buffer, methyltransferases were eluted with a linear gradient from 0 to 0.4 mol/l KCl (flow rate 72 ml/h, fraction size 5.0 ml). The KCl concentration was determined by conductivity measurements. Fractions with XMT or BMT activity were pooled separately and the enzymes precipitated with 80% ammonium sulfate. The pellet was dissolved in 20 mmol/l potassium phosphate, pH 7.0, containing 10 mmol/l 2-mercaptoethanol, dialyzed against the same buffer, and stored in 50% glycerol at -20 °C.

SAH-Sepharose. SAH-Sepharose was synthesized from ω -AH-Sepharose 4B according to Kim *et al.* [16] and equilibrated with 20 mmol/l potassium phosphate, pH 7.0, and 10 mmol/l 2-mercaptoethanol. Pooled XMT and BMT fractions from the DEAE-Cellulose step were diluted with the same buffer to a final glycerol concentration of 20% and applied to the SAH-Sepharose column (7.5×1 cm). After washing with 26 ml equilibration buffer and then with 26 ml 0.5 mol/l NaCl in the same buffer, XMT and BMT were eluted with a linear gradient from 0 to 0.5 mmol/l SAM (flow rate 24 ml/h, fraction size 2.6 ml). The SAM concentration in the effluent was followed by measuring the absorption at 260 nm. Fractions with XMT and BMT activity, respectively, were pooled, dialyzed against 20 mmol/l potassium phosphate, pH 7.0, containing 10 mmol/l 2-mercaptoethanol, concentrated by placing the dialysis tube into dry Sephadex G-100, and immediately subjected to Mono Q chromatography.

Mono Q. The XMT and BMT fractions from SAH-Sepharose chromatography were applied to a Mono Q HR 5/5 anion exchange column. The resin had been equilibrated with 20 mmol/l potassium phosphate, pH 7.0, containing 10 mmol/l 2-mercaptoethanol, and the column was washed with 7 ml of the same buffer. The enzymes were eluted from the column with a linear gradient from 0 to 0.5 mol/l KCl, increasing from 0 to 0.05 mol/l during the first min, from 0.05 to 0.3 mol/l within the next 25 min, and from 0.3 to 0.5 mol/l for another 2 min (flow rate 1 ml/min, fraction size 1 ml). Fractions containing XMT or BMT activity were pooled, dialysed against 50 mmol/l potassium phosphate, pH 7.0, containing 10 mmol/l 2-mercaptoethanol, concentrated with polyethyleneglycol (average molecular weight 100,000), and stored in 50% glycerol at -20 °C.

Gel filtration

Ascending gel filtration was performed on UL-trogel AcA 44 (1.6 × 87 cm) with 50 mmol/l potassium phosphate, pH 7.0, containing 10 mmol/l 2-mercaptoethanol (flow rate 10 ml/h, fraction size 2.5 ml).

Gel electrophoresis

Disc gel electrophoresis was performed after Davis [17]. The gel was cut longitudinally. One half was stained for protein with Coomassie Brilliant Blue [18], the other half was cut into slices of 2 mm. Each slice was tested for XMT or BMT activity by placing it into 500 µl of an assay mixture consisting of 50 mmol/l potassium phosphate, pH 7.5 for XMT and pH 8.0 for BMT, 1.5 mmol/l MgCl₂, 8.5 µmol/l [¹⁴CH₃]SAM, and 1.25 mmol/l xanthotoxol or bergapten. After incubation for 2 h at 25 °C on a shaker, the reaction was terminated by addition of 75 µl 4 N HCl. The products were extracted with 250 µl ethylacetate and quantified by liquid scintillation spectrometry.

SDS-Polyacrylamide gel electrophoresis was performed after Laemmli [19] on 10% gels. The proteins were stained with Coomassie Brilliant Blue [18].

Protein determination

Protein was measured according to Bradford [20] using Bio-Rad (München, FRG) reagents. Bovine serum albumin was used as reference.

Determination of radioactivity

Radioactivity in ethylacetate extracts of enzyme assays was measured by liquid scintillation spectrometry in a toluene-based scintillation cocktail (Rotiszint 11, Roth, Karlsruhe, FRG) after evaporation of the organic solvent. Radioactivity spots on thin-layer plates were detected by scanning (TLC-Linear Analyzer LB 2832, Berthold, Wildbad, FRG) and quantified by scraping the spots into scintillation vials followed by liquid scintillation spectrometry as above.

Results

Stimulation of furanocoumarin biosynthesis

Coumarin derivatives (Fig. 1) accumulated to maximum amounts of 160 µmol/l (equivalent to

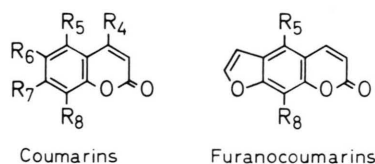
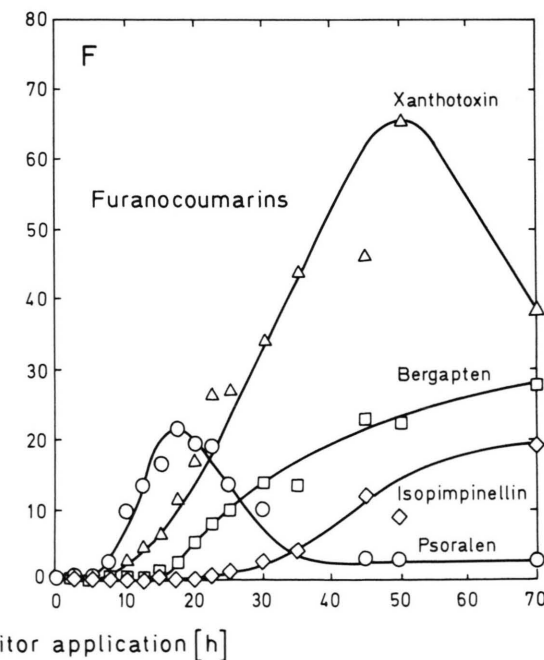
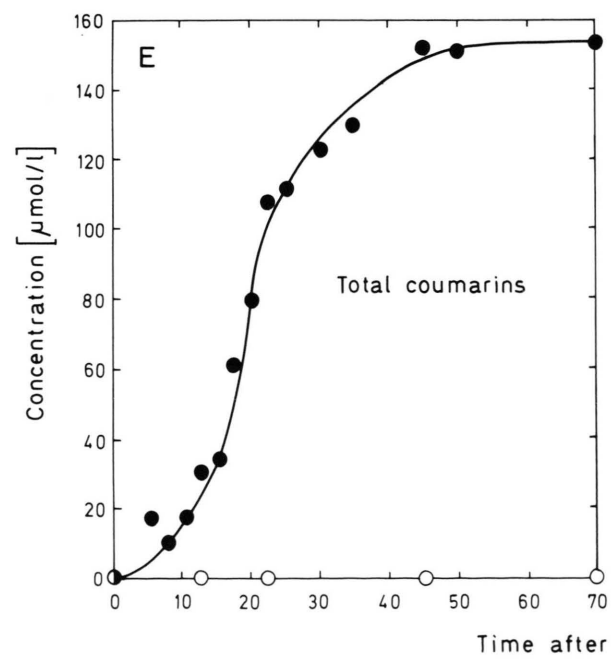
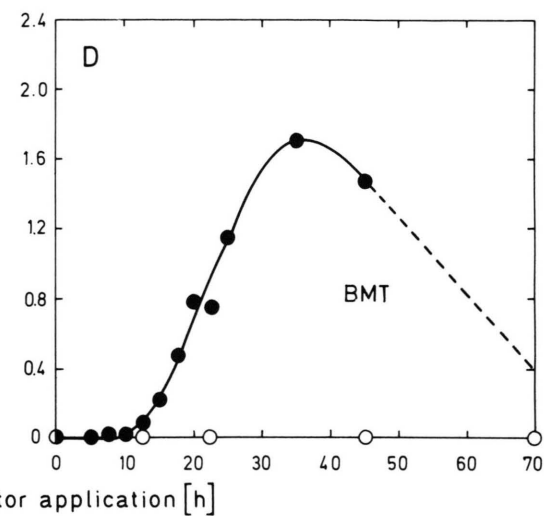
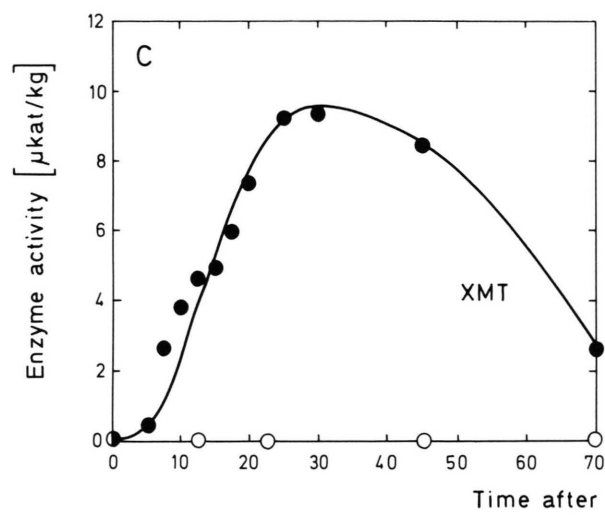
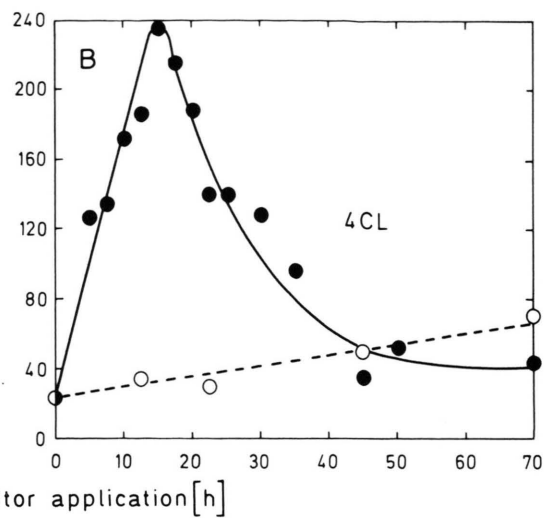
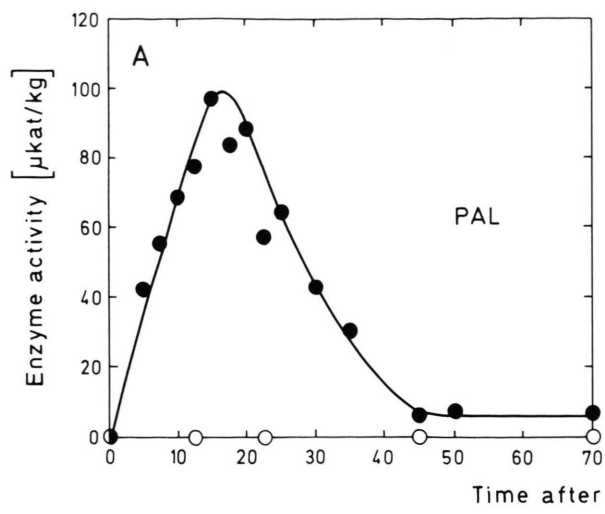


Fig. 1. Chemical structures of coumarin derivatives. **Coumarins.** 4-Hydroxycoumarin: R₅ = R₆ = R₇ = R₈ = H, R₄ = OH, umbelliferone: R₄ = R₅ = R₆ = R₈ = H, R₇ = OH, 5,7-dihydroxycoumarin: R₄ = R₆ = R₈ = H, R₅ = R₇ = OH, esculetin: R₄ = R₅ = R₈ = H, R₆ = R₇ = OH, daphnetin: R₄ = R₅ = R₆ = H, R₇ = R₈ = OH, scopoletin: R₄ = R₅ = R₈ = H, R₇ = OH, R₆ = OCH₃, hydrangetin: R₄ = R₅ = R₆ = H, R₇ = OH, R₈ = OCH₃, citropten: R₄ = R₆ = R₈ = H, R₅ = R₇ = OCH₃. **Furanocoumarins.** Psoralen: R₅ = R₈ = H, bergapten: R₈ = H, R₅ = OH, xanthotoxol: R₅ = H, R₈ = OH, bergapten: R₈ = H, R₅ = OCH₃, xanthotoxin: R₅ = H, R₈ = OCH₃, 5,8-dihydroxypsoralen: R₅ = R₈ = OH, 5-hydroxyxanthotoxin: R₅ = OH, R₈ = OCH₃, 8-hydroxybergapten: R₈ = OH, R₅ = OCH₃, isopimpinellin: R₅ = R₈ = OCH₃.

10 µmol per g cell dry weight) in the culture fluid of parsley cells treated with elicitor, whereas neither dark-grown nor illuminated cells produced these compounds (Fig. 2E). Relatively small amounts were associated with the cells. Six major components were separated by HPLC. Four of them have been identified as psoralen, xanthotoxin, bergapten, and isopimpinellin by co-chromatography with authentic samples on HPLC (isocratic and gradient systems) and TLC (solvent systems 1, 2, and 3). The results of quantifications of the identified compounds at different times after elicitor application (Fig. 2F) show that at least psoralen, and possibly also xanthotoxin, disappeared from the growth medium at later stages. This phenomenon may be due to turnover rather than re-uptake by the cells, because the amounts of cell-associated coumarins remained low during the entire period of experimentation (data not shown), while the excreted levels increased drastically.

Transient increases in XMT and BMT activities correlated well with the appearance of the products in the culture fluid. The activity of XMT increased several hours earlier than that of BMT, and a similar time shift was observed for the respective products (Fig. 2C, D, and F). On the other hand, the increases in these two enzyme activities occurred significantly later than the previously observed [7] coordinated increases in the activities of PAL and 4CL (Fig. 2A, B, C, and D), which increased almost immediately after elicitor addition, reached maximum



levels at about 15 h, and then decreased rapidly to very low levels. The activities of XMT and BMT increased only after apparent lag phases of approximately 5 and 10 h with peaks around 30 and 25 h, respectively.

The highest level of psoralen, the last common precursor of xanthotoxin, bergapten, and isopimpinellin [1], was observed 18 h after the addition of elicitor, shortly after the activity peaks of PAL and 4CL (Fig. 2A, B, and F).

Purification of XMT and BMT

A number of hydroxylated coumarin derivatives was tested as substrates for O-methyltransferase activities in crude extracts of parsley cells harvested 35 h after elicitor treatment. With the exception of umbelliferone and scopoletin, all of the tested com-

pounds were methylated to different degrees. High conversion rates were observed for xanthotoxin and bergapten (see also below), which were therefore used as substrates in the following experiments.

The purification procedures for XMT and BMT are summarized in Tables I and II. Both enzymes were completely separated from CMTs on DEAE-Cellulose (Fig. 3). The most efficient purification step for XMT (Table I) and BMT (Table II) was affinity chromatography on SAH-Sepharose. The two enzymes were finally separated from each other by anion exchange chromatography on Mono Q (Fig. 4). After analytical disc gel electrophoresis XMT and BMT activities were associated each with a major protein band (Fig. 5). One major contaminating protein without enzyme activity was also visible on each gel. SDS-Polyacrylamide gels, which were run after each purification step, showed that XMT

Table I. Purification procedure for XMT.

Purification step	Volume [ml]	Total enzyme activity [pkat]	Protein [mg]	Specific enzyme activity [μ kat/kg]	Yield [%]	Purification factor [-fold]
Crude extract	1,155	4,550	1,028	4.4	100	1
Ammonium sulfate precipitate (40–70%), desalted	65	5,868	512	11.5	129	2.6
DEAE-Cellulose	5	2,571	80	31.9	57	7
SAH-Sepharose	12.5	1,347	2.9	465	30	106
Mono Q	6.7	621	0.24	2,576	14	580

Table II. Purification procedure for BMT.

Purification step	Volume [ml]	Total enzyme activity [pkat]	Protein [mg]	Specific enzyme activity [μ kat/kg]	Yield [%]	Purification factor [-fold]
Crude extract	1,155	2,183	1,028	2.1	100	1
Ammonium sulfate precipitate (40–70%), desalted	65	2,668	512	5.2	122	2.5
DEAE-Cellulose	4.4	1,115	44	25.5	51	12
SAH-Sepharose	7.5	406	2.9	140	19	66
Mono Q	7.8	192	0.28	685	9	323

- ◀ Fig. 2. Time courses of elicitor-stimulated changes in enzyme activities related to furanocoumarin biosynthesis and of product accumulation in parsley cell suspension cultures. Specific enzyme activities of PAL (A), 4CL (B), XMT (C) and BMT (D), and total coumarin derivatives (E) were monitored at the indicated times after elicitor treatment (closed circles) in comparison with untreated cultures (open circles). The changes in concentration of four individual furanocoumarins in the culture fluid of elicitor-treated cells were quantified by HPLC analysis (F). Each point in A, B, E and F represents the mean value from three independent experiments. Because of large variations in XMT and BMT activities between different experiments, representative results from one individual experiment are shown in C and D.

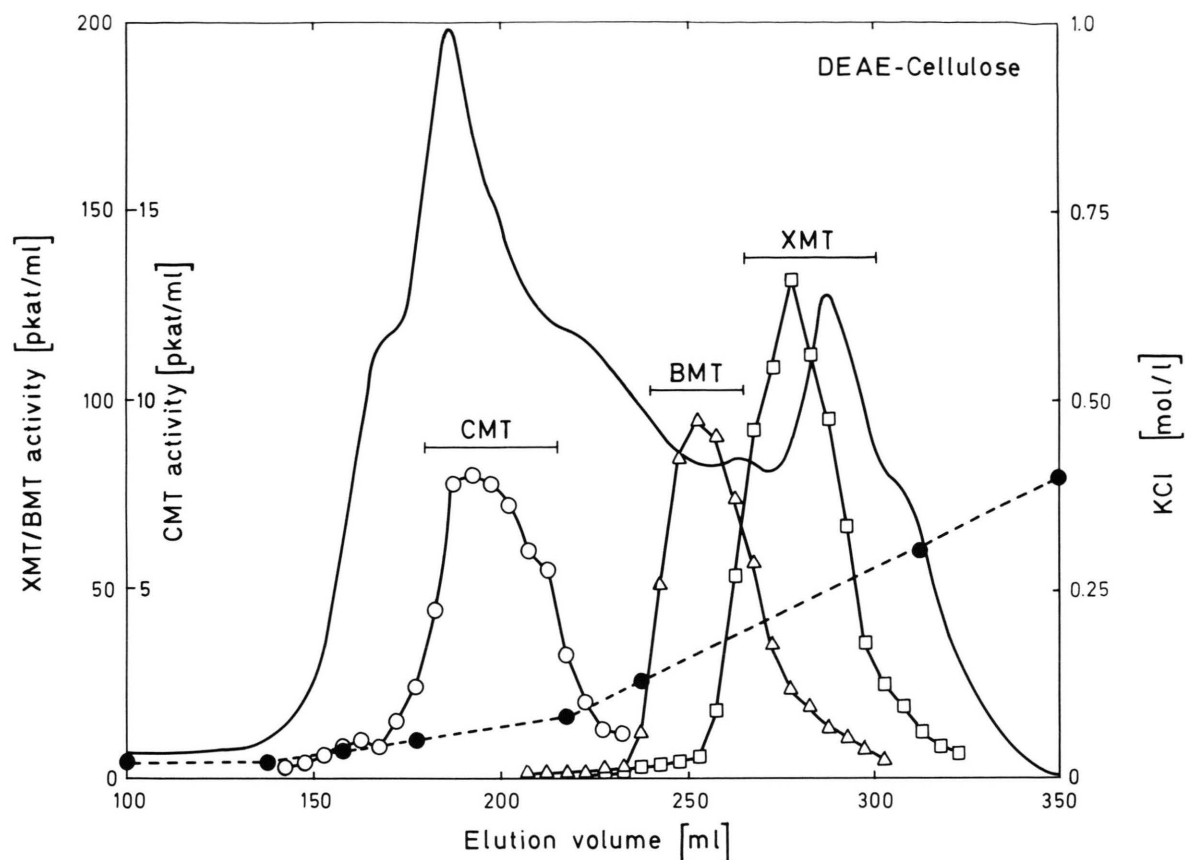


Fig. 3. DEAE-Cellulose elution profile of desalted ammonium sulfate precipitate. All enzyme activities were monitored over the whole elution volume, but zero activities were not included in the drawing (— absorption at 280 nm, ●---● KCl concentration, ○—○ CMT, △—△ BMT, □—□ XMT). Bars indicate fractions pooled for further purification.

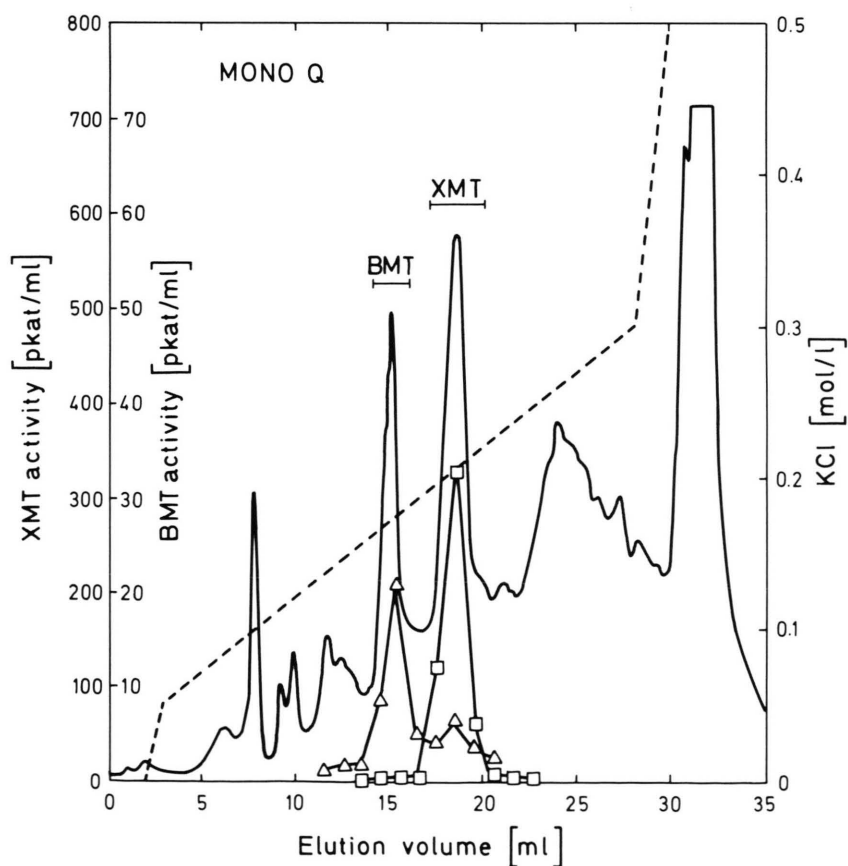


Fig. 4. Mono Q elution profile of combined XMT and BMT fractions from DEAE-cellulose chromatography. The whole effluent was assayed for CMT, XMT and BMT activities, but zero activities were not included in the drawing (— absorption at 280 nm, --- KCl concentration, △—△ BMT, □—□ XMT). Bars indicate fractions pooled for further experiments.

and BMT purification resulted in the preferential enrichment of M_r -34,000 and M_r -36,000 proteins, respectively (Fig. 6). The following experiments were carried out with these enzyme preparations.

Properties of XMT and BMT

pH Optima. The pH optima were determined using different buffers of the same ionic strength but different pH. XMT and BMT showed maximum activities at pH 7.5–8.0 and 8.0–8.5, respectively (Fig. 7).

Stability. After ammonium sulfate precipitation and dialysis, XMT and BMT did not lose activity during storage for 20 days in 50% glycerol at -20°C . 2-Mercaptoethanol in the enzyme assays did not change the activity, but stabilized both enzymes during purification and storage for prolonged periods of time.

Effects of buffer conditions. The methylation rate of bergapton by BMT was not affected by buffer concentration, whereas XMT activity decreased drastically with increasing concentrations of potassium phosphate from 1.25 mmol/l to 1.0 mol/l (Fig. 8). Both enzymes were inactivated within less than 30 min in buffers below pH 5. Therefore further purification by chromatofocussing was not successful.

Molecular mass. Apparent molecular masses of 67,000 for XMT and 73,000 for BMT were determined by gel filtration (Fig. 9), in contrast to about twofold lower values obtained under denaturing conditions (see above). This might indicate the possibility that the native enzymes consist of two subunits each.

Substrate specificities. The relative conversion rates of a number of coumarin derivatives by the

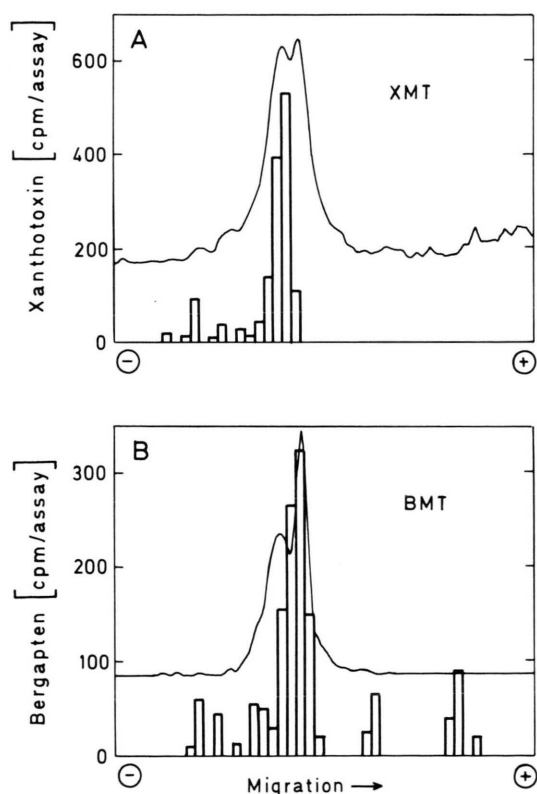


Fig. 5. Disc gel electrophoresis of purified XMT (A) and BMT (B). The columns represent enzyme activities in individual gel slices and the line indicates Coomassie Brilliant Blue stained proteins after scanning with a Laser Densitometer 2202 Ultrosan (LKB, Gräfelfing, FRG).

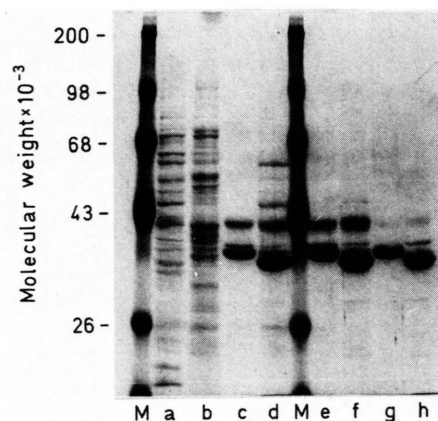


Fig. 6. SDS-Polyacrylamide gel electrophoresis of BMT (a, c, e, g) and XMT (b, d, f, h) at different stages of purification (DEAE-Cellulose: a, b; SAH-Sepharose: c, d; gel filtration: e, f; Mono Q: g, h). Ten μg of protein was applied to lanes a–f and 5 μg to lanes g and h. Proteins were stained with Coomassie Brilliant Blue. M = Molecular weight markers.

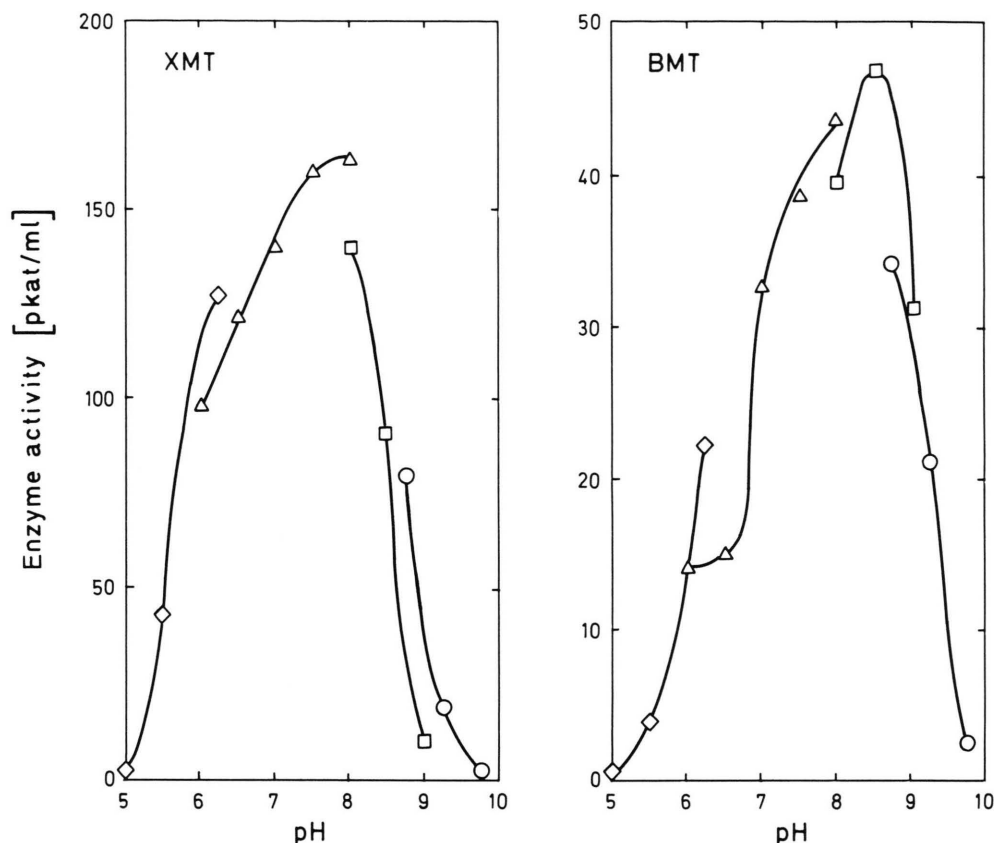


Fig. 7. pH Optima of purified XMT and BMT. The following buffer systems were used at identical ionic strengths, based on 50 mmol/l potassium phosphate at pH 7.0; ◇—◇ citrate/NaOH, △—△ potassium phosphate, □—□ Tris/HCl, ○—○ glycine/NaOH.

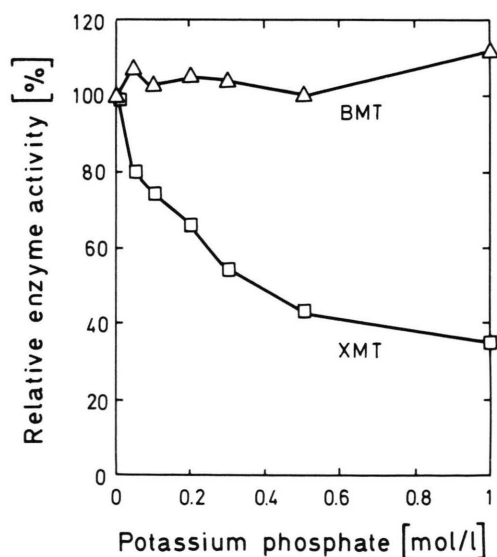


Fig. 8. Effects of potassium phosphate concentration (at pH 8.0) on XMT and BMT activities.

purified enzymes in comparison with the reactions in the crude extract are listed in Table III. Only furanocoumarins were accepted by XMT and BMT, whereas caffeate, 2,4,6-trihydroxybenzoate, quercetin, and kaempferol were not methylated. In addition to xanthotoxol, the 8-hydroxy groups of daphnetin and 8-hydroxybergapten were methylated by XMT, though only at relative rates lower than 2%. BMT specifically catalyzed the methylation of 5-hydroxy groups of furanocoumarins. 5-Hydroxyxanthotoxin was methylated at an even higher rate than bergaptol. Both hydroxy groups of dihydroxylated coumarin derivatives, such as 5,7-dihydroxycoumarin and 5,8-dihydroxypsoralen, were methylated by BMT, provided one hydroxy group was in the 5-position. Since both enzymes were not purified to complete homogeneity, it cannot be excluded that methylation of bergaptol and 5-hydroxyxanthotoxin by XMT as well as methylation of xanthotoxol and 8-

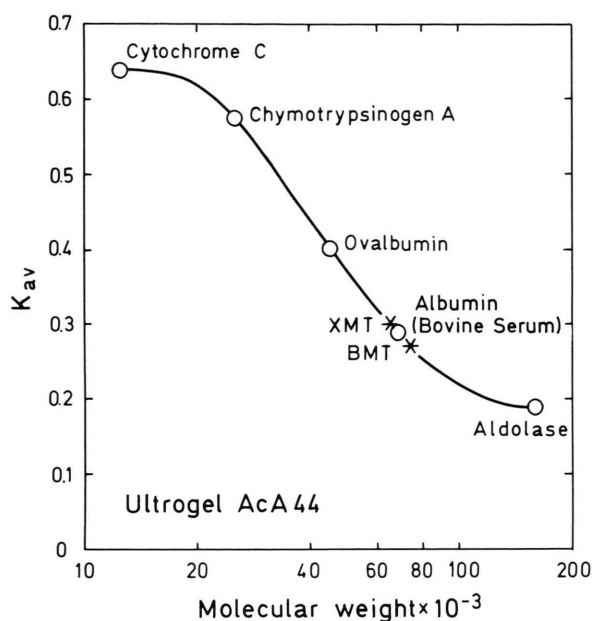


Fig. 9. Estimation of apparent molecular masses of XMT and BMT by gel filtration in the presence of the indicated reference proteins.

hydroxybergapten by BMT was due to residual cross-contamination.

Xanthotoxin was the only product formed by XMT, when xanthotoxol was used as substrate either alone or in the presence of 8-hydroxybergapten (Table IV). 8-Hydroxybergapten as sole substrate of XMT was converted to isopimpinellin with 2% of the efficiency observed with xanthotoxol, although this reaction occurred at higher rates in crude cell extracts (Table III). Therefore, a third methyltransferase besides XMT and BMT may be involved in the biosynthesis of isopimpinellin via bergapten. When bergapten and 5-hydroxyxanthotoxin were applied simultaneously to BMT, only 5-hydroxyxanthotoxin was converted at rates similar to those observed with this substrate alone (Table V).

Apparent K_m and V_{max} values were determined for all major substrates at concentrations where no product inhibition occurred. The values calculated after Eadie [21] and Hofstee [22] are summarized in Table VI. Almost identical values resulted from calculations on the basis of Lineweaver/Burk or Hanes plots [23, 24].

Table III. Relative methylation rates by XMT and BMT, as compared with crude cell extract.

Substrate ^a	Product ^b	Relative conversion rate [%]		
		XMT	BMT	Crude extract
COUMARINS				
4-Hydroxycoumarin	—	0	0	n.d. ^c
Umbelliferone	—	0	0	0
5,7-Dihydroxycoumarin	citropten ^d	0	6	6
Esculetin	scopoletin ^e	0	0	0.6
Daphnetin	hydrangetin	0.6	0	0.6
Scopoletin	—	0	0	0
FURANOCOUMARINS				
Bergaptol	bergapten	0.5	100	14.8
Xanthotoxol	xanthotoxin	100	1	100
5,8-Dihydroxypsoralen	isopimpinellin	0	2.6	37.4
5-Hydroxyxanthotoxin	isopimpinellin	0.7	125	9.7
8-Hydroxybergapten	isopimpinellin	2	0.8	9.7

^a Substrate concentrations were in the range from 0.2 to 0.25 mmol/l. 5,8-Dihydroxypsoralen was assayed in the presence of 5 mmol/l Na_2SO_3 , and the relative conversion rate was calculated in comparison to assays with xanthotoxol and bergapten containing the same concentration of Na_2SO_3 .

^b Reaction products were identified by co-chromatography with authentic samples on TLC using solvent systems 1, 2, and 3.

^c n.d. = not determined.

^d Three products were detected on TLC using solvent system 1. A large portion (40%) of the radioactivity co-chromatographed with authentic citropten. Two additional products accounted for 30% each.

^e In addition to scopoletin, a second unidentified product was formed.

Table IV. Relative activity and reaction products of XMT in the presence of either one or two substrates.

Substrates		Product ^a	Relative enzyme activity [%]
Xanthotoxol [mmol/l]	8-Hydroxybergapten [mmol/l]		
0.20	0	xanthotoxin	100
0	0.25	isopimpinellin	2
0.20	0.25	xanthotoxin	82
0.08	0.15	xanthotoxin	101

^a Reaction products were identified by co-chromatography with authentic samples on TLC using solvent systems 1, 2, and 3.

Table V. Relative activity and reaction products of BMT in the presence of either one or two substrates.

Substrates		Product ^a	Relative enzyme activity [%]
Bergaptol [mmol/l]	5-Hydroxyxanthotoxin [mmol/l]		
0.25	0	bergapten	100
0	0.25	isopimpinellin	125
0.25	0.25	isopimpinellin	125
0.15	0.10	isopimpinellin	115
0.05	0.20	isopimpinellin	118

^a Reaction products were identified by co-chromatography with authentic samples on TLC using solvent systems 1, 2, and 3.

Table VI. Apparent K_m and V_{max} values for major substrates of XMT and BMT.

Enzyme	Substrates [μmol/l] Constant	Variable	K_m [μmol/l]	V_{max} [μkat/kg]
XMT	SAM (17.9)	xanthotoxol (5.0–1,000)	9.8	5,750
	xanthotoxol (1,000)	SAM (2.1–35)	4.4	7,480
BMT	SAM (8.5)	bergaptol (2.5–500)	4.0	980
	bergaptol (250)	SAM (2.1–35)	3.1	1,160
	SAM (13.4)	5-hydroxyxanthotoxin (1.25–250)	1.0	1,250
	5-hydroxyxanthotoxin (250)	SAM (2.2–29)	3.1	1,800

Discussion

Xanthotoxin, bergapten, isopimpinellin, psoralen, and graveolone were first identified in the culture medium of elicitor-treated parsley cells by Tietjen *et al.* [6]. In their study, isopimpinellin was not synthesized in response to treatment with the elicitor used here. This is in contrast to our present results and may be explained by the use of different parsley

cell cultures [11] and/or by the high dose of elicitor applied in their experiments, which significantly reduces the amount of coumarins accumulated in the medium [13].

The previously observed sequential activity changes in elicitor-treated parsley cells of PAL, C4H and 4CL on the one hand, and DUD on the other hand [7–9] have now also been demonstrated for

XMT and BMT relative to PAL and 4CL. The relative timing of these enzyme activity changes is consistent with the transient accumulation of psoralen in our studies, where this compound was first detectable in the culture fluid shortly after the activity increases of PAL and 4CL. In accordance with its suggested role as a common precursor of xanthotoxin, bergapten, and isopimpinellin [1], psoralen accumulated prior to the activity increases of XMT and BMT, both of which are concluded from these studies to catalyze final steps in the biosynthesis of these products. Thus, all of the so far identified biosynthetic enzymes of the three methylated furanocoumarins are stimulated by the elicitor treatment in the same order as they are needed for their synthesis. The possible role of CMT activities in the furanocoumarin or other phenylpropanoid pathways in this system has not been studied in detail.

Anion exchange by FPLC proved to be a very efficient method for the separation of XMT and BMT from parsley cells. Similar rates of purification and the complete separation of XMT and BMT from *Ruta graveolens* have previously been achieved by general ligand affinity chromatography [25]. The enzymes from *Ruta* have similar pH optima, but differ significantly in their apparent molecular masses from XMT and BMT from parsley [26]. Similar high substrate specificities were observed for the enzymes from both species. XMT, but not BMT, from *Ruta graveolens* was eluted with xanthotoxol from the af-

finity column, whereas bergaptol released only BMT [25].

The results of mixed substrate experiments with the purified enzymes from parsley suggest that XMT exclusively catalyzes the methylation of xanthotoxol, whereas BMT methylates both, bergaptol and 5-hydroxyxanthotoxin. Therefore, an additional furanocoumarin O-methyltransferase might be responsible for the methylation of 8-hydroxybergapten in crude extracts of parsley. Application of different potential substrates to crude extracts from cell cultures of *Ruta graveolens* [26] and parsley as well as precursor feeding to *Ruta* cell cultures [27] suggest that isopimpinellin may be synthesized *via* 5-hydroxyxanthotoxin, 8-hydroxybergapten or 5,8-dihydroxypsoralen, although the route *via* 5-hydroxyxanthotoxin seems to predominate.

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